Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by N-methyl-D-aspartate receptor activation

Marchetti, L; Klein, M; Schlett, K; Pfizenmaier, K; Eisel, ULM

Published in:
The Journal of Biological Chemistry

DOI:
10.1074/jbc.M311766200

Publication date:
2004

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 31, Issue of July 30, pp. 32869–32881, 2004
Printed in U.S.A.

Tumor Necrosis Factor (TNF)-mediated Neuroprotection against Glutamate-induced Excitotoxicity Is Enhanced by *N*-Methyl-D-aspartate Receptor Activation

ESSENTIAL ROLE OF A TNF RECEPTOR 2-MEDIATED PHOSPHATIDYLINOSITOL 3-KINASE-DEPENDENT NF-κB PATHWAY*

Received for publication, October 27, 2003, and in revised form, May 14, 2004
Published, JBC Papers in Press, May 21, 2004, DOI 10.1074/jbc.M311766200

Lara Marchetti‡§, Matthias Klein‡¶, Katalin Schlett‡‖, Klaus Pfizenmaier‡‡‡, and Ulrich L. M. Eisel‡ ‡‡§§**

From the ‡Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, D-70569 Stuttgart, Germany, ¶Xantos Biomedicine AG, Fraunhoferstrasse 22, D-82152 Martinsried, Germany, ‖Department of Physiology and Neurobiology, Eötvös Loránd University, Pázmány P. stny. 1/C, H-1117 Budapest, Hungary, and §§Department of Molecular Neurobiology, Rijksuniversiteit Groningen, Kerklaan 30, P. O. Box 14, 9750 AA Haren, The Netherlands

We have previously shown that two tumor necrosis factor (TNF) receptors (TNFR) exhibit antagonistic functions during neurodegenerative processes *in vivo* with TNFR1 aggravating and TNFR2 reducing neuronal cell loss, respectively. To elucidate the neuroprotective signaling pathways of TNFR2, we investigated glutamate-induced excitotoxicity in primary cortical neurons. TNF-expressing neurons from *TNF*-transgenic mice were found to be strongly protected from glutamate-induced apoptosis. Neurons from wild type and *TNFR1*$^{-/-}$ mice prestimulated with TNF or agonistic TNFR2-specific antibodies were also resistant to excitotoxicity, whereas *TNFR2*$^{-/-}$ neurons died upon glutamate and/or TNF exposures. Both protein kinase B/Akt and nuclear factor-κB (NF-κB) activation were apparent upon TNF treatment. Both TNFR1 and TNFR2 induced the NF-κB pathway, yet with distinguishable kinetics and upstream activating components, TNFR1 only induced transient NF-κB activation, whereas TNFR2 facilitated long term phosphatidylinositol 3-kinase-dependent NF-κB activation strictly. Glutamate-induced triggering of the ionotropic *N*-methyl-D-aspartate receptor was required for the enhanced and persistent phosphatidylinositol 3-kinase-dependent NF-κB activation by TNFR2, indicating a positive cooperation of TNF and neurotransmitter-induced signal pathways. TNFR2-induced persistent NF-κB activity was essential for neuronal survival. Thus, the duration of NF-κB activation is a critical determinant for sensitivity toward excitotoxic stress and is dependent on a differential upstream signal pathway usage of the two TNFRs.

Tumor necrosis factor (TNF)[1] is a prominent proinflammatory mediator that has been causally associated with the pathophysiology of several acute and chronic diseases, in particular rheumatoid arthritis and Morbus Crohn (1, 2). Up-regulated TNF expression has also been found in various neurodegenerative diseases such as cerebral malaria, AIDS dementia, Alzheimer's disease, multiple sclerosis, and stroke, suggesting a potential pathogenic role of TNF in these diseases as well (3–7). The membrane-expressed form of TNF signals through both TNF receptors (TNFR1 and TNFR2), whereas soluble TNF proteolytically cleaved from the membrane form acts mainly via TNFR1 (8). Signal pathways initiated from the death domain-containing TNFR1, leading to both proapoptotic and anti-apoptotic cellular responses, have been studied in great detail (9). In contrast, there is less information regarding the molecular mechanisms surrounding signal pathways and cellular responses solely initiated via TNFR2 because of concomitant TNFR1 signals in normal situations. The evaluation of the physiological role of TNFR2 by large depends on data obtained from *TNFR1*$^{-/-}$ mice.

We have recently investigated the role of TNF and its receptors in retinal ischemia and unraveled an antagonistic function of TNFR1 and TNFR2. TNFR2 exerts neuroprotection in a phosphatidylinositol 3-kinase (PI3K) dependent manner, which is counterbalanced by the neurodegenerative action of TNFR1 (10). TNFR1 has also been associated with the cell death of hippocampal neurons responding to TNF (11), whereas inhibition of TNFR2 expression by the use of antisense oligonucleotides sensitized neuronal-like cells toward apoptosis (12). To elucidate the neuroprotective signal pathways emanating from TNFR2, we have established an *in vitro* model of apoptosis induction of primary cortical neurons by glutamate-triggered excitotoxicity to study mechanisms controlling apoptosis sensitivity of neurons within wild type (C57BL/6) and *TNF* transgenic *TNFR1*$^{-/-}$ and *TNFR2*$^{-/-}$ mice. Our data reveal, for the first time, differential activation kinetics of NF-κB and differential usage of upstream signaling components by TNFR1 and TNFR2. TNFR2, but not TNFR1, induces persistent NF-κB activation via a signaling pathway involving PI3K and PKB/Akt, which is strongly enhanced by *N*-methyl-D-aspartate receptor co-stimulation.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ A recipient of a Landesgraduiertenförderung stipend from the state of Baden-Wuerttemberg.

** To whom correspondence should be addressed. Tel.: 49-711-685-6986; Fax: 49-711-685-7484; E-mail: klaus.pfizenmaier@izi.uni-stuttgart.de.

‡‡ Supported by grants from Volkswagen Foundation, EU Commission, Hertie Foundation, and the Deutsche Forschungsgemeinschaft (SFB 495).

[1] The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; UTR, untranslated region; NF-κB, nuclear factor-κB; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; NR2B, NMDAR subunit 2B; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; PBS, phosphate-buffered saline; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; IKK, IκB kinase; ANOVA, analysis of variance.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice with Brain-specific TNF Expression—Transgenic mice were developed by pronuclear injection in general as previously described (13). A construct consisting of the murine NMDAR subunit 2B (NR2B) promoter and the first three exons of the 5′-untranslated region (UTR) of the *NR2B* gene was fused with the NarI site of the first exon of the murine *TNF* gene with the replaced 3′-UTR (3′ part of exon 4 of the *TNF* gene) by the 3′-UTR of the human *β-globin* gene (14). Four mouse lines were investigated and showed similar TNF expression patterns differing only in the level of and duration of expression. The expression pattern was verified by *in situ* hybridization as described previously (15). The pBSII-SK plasmid containing a cDNA of the murine *TNF* gene was digested either with PstI or EcoRI for synthesis of a ^{35}S-labeled sense or antisense probe using T3 or T7 RNA polymerase.

Primary Cortical Cell Culture—Cortical cultures were prepared as follows. Embryonic brains were extracted (embryonic day 14), the meninges were removed, and the cortices were dissected immediately. Neurons were recovered by mechanical dissociation. Cells were plated at a density of ~1×10^5 cells/cm^2 (96- or 6-well plates) or 2.5×10^4 cells/cm^2 (24-well plates) on poly-L-lysine (Sigma) coated plates. Neurobasal medium with B27 supplement (Invitrogen) and 0.5 mM glutamine with a final concentration of 0.1 mg/ml gentamycin (Invitrogen) and 2.5 μg/ml amphotericin B (Sigma) was used as culture medium. On the second day *in vitro*, cells were treated with 10 μM cytosine arabinoside for 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged, and every third day afterward, fresh medium was added.

Cells were either stimulated with increasing concentrations of glutamate for 1 h (Figs. 2*B*, 3, *A* and *C*, and 8*C*) or for 24 h with the indicated concentrations of TNF (Figs. 3*B* and 8, *A* and *B*) or TNFR agonistic antibodies (Fig. 4, *A–C*) followed by a 1-h stimulation of 250 μM glutamate (Sigma) and the determination of the cell viability 24 h later. When indicated, cells were incubated prior to the glutamate stimulus with an inhibitor of PI3K (LY294002 (25 μM), Calbiochem, or wortmannin (100 nm), Sigma), NMDAR (MK801 (10 μM), Tocris), caspases (Z-VAD-fmk (20 μM), BACHEM), or NF-κB (MG-132 (20 μM), Calbiochem; geldanamycin (0.5 μM), Calbiochem; or BAY11-7082 (20 μM), Alexis Biochemicals). To determine the kinetics of cell death induction, primary cortical neurons were incubated for 1 h with 250 and 500 μM glutamate and cell viability was assessed directly after the stimulus (=1-h) or 1 (=2-h), 3 (=4-h), 5 (=6-h), and 23 (=24-h total incubation time) h later (Fig. 2*A*). Agonistic antibodies (2 μg/μl; HyCult Biotechnology) were cross-linked by a mouse anti-rat antibody (2 μg/μl; Jackson ImmunoResearch, Dianova, Hamburg, Germany) to obtain optimum triggering of the TNFRs.

Determination of Cell Viability—Cell viability was determined by the colorimetic MTT (3-(4,5-dimethylthiazol-2-yl-)2,5-diphenyltetrazolium bromide) assay as described previously (16). Primary cortical neurons were grown in 96-well microtiter plates at a density of 6×10^4 cells/well and stimulated as indicated. 10 μl of an MTT solution (1.25 mg/ml) was added to each well. After a 1-h incubation, cells were lysed by adding 150 μl of isopropyl-HCl solution (600 μl of HCl/100 ml isopropyl alcohol) for 15 min. The absorbance of each well was determined with an automated ELISA reader (Bio-Rad) at 590 nm with a background correction at 620 nm.

Western Blot—Cytosolic extracts from primary cortical neurons (2×10^6) were prepared by washing twice with ice-cold phosphate-buffered saline (PBS) followed by the addition of 0.4 ml of the cell lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride). Lysates were incubated on ice for 20 min. 25 μl of 10% Nonidet P-40 was added for 2 min, and nuclei were collected by centrifugation. The supernatants were boiled for 5 min in Laemmli's sample buffer (2% SDS, 5% DTT), and 50 μg of protein was separated by SDS-polyacrylamide gel electrophoresis. After transfer to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences), proteins were detected with a specific primary and a horseradish peroxidase-conjugated secondary antibody using enhanced chemiluminescence according to the manufacturer's instructions (LuminGlo, New England Biolabs).

Primary antibodies included were a rabbit polyclonal antibody specific for the phospho-PKB/Akt serine 473 residue (1:1000; Cell Signaling), total PKB/Akt (1:1000; Cell Signaling), IκB (1:500; Santa Cruz Biotechnology), phospho-IκB (1:1000; Cell Signaling), TNF (1:1000; BIOSOURCE), and glutamate receptor 1 (1:300; Chemicon).

Immunocytochemistry and Immunohistochemistry—Cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 20 min at room temperature, permeabilized with 0.1% Triton X-100/PBS for 5 min, and blocked with 5% goat serum in PBS. Overnight incubation with a polyclonal rabbit anti-p65 antibody (1:500; Oncogene) or rabbit anti-TNF antibody (1:1000, Santa Cruz Biotechnology) at 4 °C was followed by a 1-h incubation with a fluorescent-labeled Alexa 546 or 488 anti-rabbit antibody (1:1000; Molecular Probes) at room temperature. Neurons were labeled with a monoclonal neuronal-specific mouse anti-unique-β-tubulin antibody (1:2000; Babco) for 1.5 h at room temperature and a Alexa 488 or 546 anti-mouse antibody (1:1000).

Glia cells were detected using a monoclonal mouse anti-glial fibrillary acidic protein antibody (1:2000; Sigma) and a secondary Alexa 546-labeled anti-mouse antibody (1:1000). The glia cell content resulted in <0.5%.

Determination of apoptosis induction was assessed after fixation of stimulated (1 h 250 μM glutamate) and unstimulated cells by incubation with an annexin V-specific fluorescein isothiocyanate-conjugated antibody (1:20; BD Biosciences) for 1 h at room temperature followed by a 1-h incubation with DAPI (0.1 μg/ml) for nuclear staining,

For immunohistochemical staining, paraffin-embedded sections were permeabilized with 0.02% H_2O_2-methanol for 30 min and blocked with 10% fetal calf serum for 20 min. Sections then were incubated with specific antibodies (rabbit-anti-TNF (Genzyme) at 1:1000; rat-anti-mac3 (BD Biosciences) at 1:200; and rat-anti-CD45 (BD Biosciences) at 1:100 in 0.1% bovine serum albumin/Tris-buffered saline) at 4 °C overnight, washed, incubated with a biotin-labeled anti-rabbit IgG or anti-rat-IgG (Vector Laboratories at 1:200 in 2.5% bovine serum albumin/Tris-buffered saline), and stained with avidin-diaminobenzidine (Sigma) according to the manufacturer's protocol.

Electrophoretic Mobility Shift Assay (EMSA)—For preparation of nuclear extracts, 2×10^6 primary cortical neurons were washed once with cold PBS, resuspended in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) and then incubated on ice for 20 min. Subsequently, 25 μl of 10% Nonidet P-40 was added for 2 min, and nuclei were collected by centrifugation and resuspended in 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride, shaken for 20 min, and then centrifuged. The supernatant containing nuclear proteins was used for EMSA. The NF-κB consensus oligonucleotide (5′-ATCAGGGACTTTC-CGCTGGGGACTTTCCG-3′) was 5′-labeled with [γ-^{32}P]ATP using polynucleotide kinase. Binding reaction was performed by incubating 10 μg of nuclear protein extracts with 4 μg of poly(dI-dC) in a 20-μl reaction mixture containing 5 mM $MgCl_2$, 50 mM KCl, 5 mM HEPES, pH 7.8, 0.2 mM EDTA, 5 mM DTT, and 10% glycerol. The double-stranded oligonucleotide probe (50,000 cpm/μl) was added for 20 min at room temperature. Binding specificity was determined by competition with excess (100 ng) unlabeled NF-κB oligonucleotide. DNA/protein complexes were analyzed on non-denaturing 6% polyacrylamide gels and visualized by PhosphorImaging. For supershift assays, nuclear extracts were preincubated with p65, p50, or cRel antibodies (1 μg/ml; Santa Cruz Biotechnology) for 30 min at room temperature.

RESULTS

Generation and Analyses of NR2B/TNF Transgenic Mice—Previous transgenic mouse models resolving the role of TNF in the brain were designed to cause a widespread and often high level of TNF expression, which was typically associated with various pathologies (17–20). To avoid unconditional TNF expression, we have employed the promoter of the murine NMDA receptor subunit *NR2B* gene to generate mice with a strictly regional and rather moderate neuronal TNF expression. The endogenous expression of NR2B subunit is restricted to forebrain regions and mainly expressed in cortical and hippocampal regions of the brain (21, 22). To enforce this restricted expression pattern of TNF in transgenic mice, the 5′-UTR of the *NR2B* gene, which was shown to influence the expression pattern, was also included in the construct (Fig. 1*A*) (20, 23). *NR2B*-directed *TNF* transgene expression was verified by *in situ* hybridization (Fig. 1*B*), Western blot analyses in embryonic day 14 and postnatal day 14 tissues (Fig. 1*C*), immunolabeling of primary neuronal cells (Fig. 1, *D* and *E*), and immunostaining in paraffin sections (Fig. 1*F*, *upper panel*). Interestingly, among the four generated mouse lines, no in-

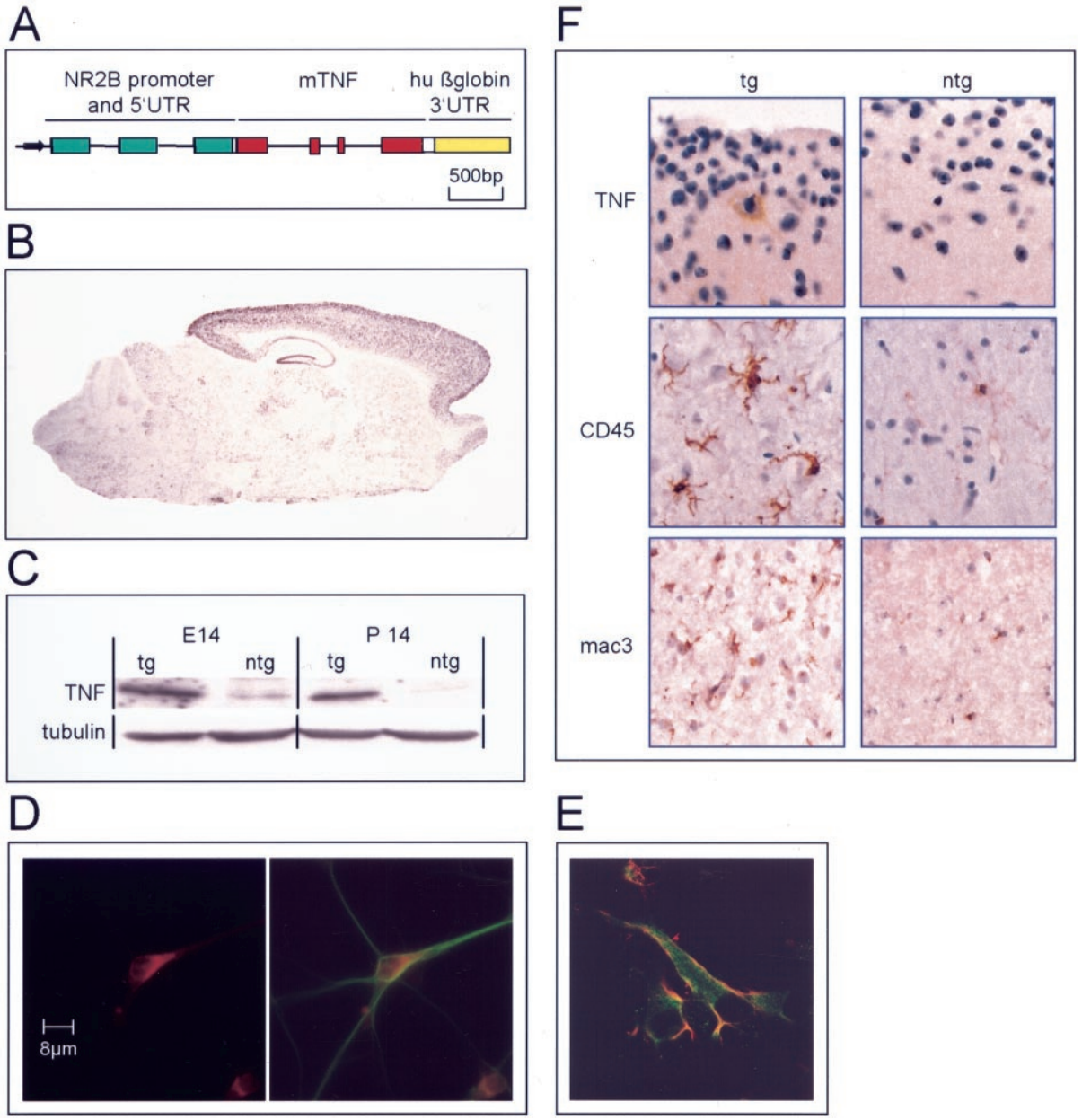

FIG. 1. **Generation of NR2B/TNF transgenic mouse lines.** *A*, schematic depiction of the transgene construct. The promoter and 5′-UTR of the NMDA receptor subunit *NR2B* gene (exons 1–3 in *green boxes*) was fused to the NarI site of the murine *TNF* gene (*red boxes*) in exon 1, thus replacing part of its 5′-UTR. The 3′-UTR of the murine *TNF* gene was replaced by the human *β-globin* gene (*yellow box*) (14) for stabilizing the transgenic message, resulting in a 4.9-kb DNA fragment, which was used for pronuclear injection. *B*, *in situ* hybridization. 10 μm of semi-thin cryostat midsagittal sections were hybridized with a ^{35}S-labeled TNF-antisense probe to detect TNF expression in the brain of transgenic mice (three months of age). In contrast to non-transgenic controls, transgene expression could be detected in forebrain regions, mainly in the cortex and hippocampus resembling the expression pattern of the *NR2B* gene. *C*, Western blot. Detection of TNF expression in embryonic (embryonic day 14) and postnatal cortical tissue (postnatal day 14) of *NR2B/TNF* transgenic (*tg*) animals in comparison to non-transgenic (*ntg*) control. *D*, immunocytochemistry. TNF expression was detected by a rabbit anti-TNF antibody (*red fluorescence*, Alexa 546) in *in vitro* cultivated cortical neurons by video microscopy. *Left picture* shows TNF expression alone, and the *right picture* shows TNF expression merged with βIII-tubulin staining (*green fluorescence*, Alexa 488) to mark neuronal cell bodies. *E*, confocal section of TNF-stained (*red fluorescence*, Alexa 546) and tubulin-stained (*green fluorescence*, Alexa 488) cortical neurons to verify membrane expression of TNF. *F*, immunohistochemistry. 1-month-old tg and ntg cortex was stained for TNF in the *upper panel*. Staining for CD45 (*middle panel*) and mac3 (*lower panel*) indicates activated microglia in TNF transgenic cortices.

flammatory responses such as infiltration or spontaneous demyelinating lesions were observed as reported from other mouse models with ubiquitous and high level of TNF expression (17–20). Only in areas of strongest TNF expression (*i.e.* hippocampus and cortex), ramified microglial cells positive for CD45 or mac3, considered as activation markers, could be detected (Fig. 1*E*, *middle* and *lower panels*). The data show that *TNF* transgene expression is in concordance with the expected pattern of the *NR2B* gene (22, 23). Moreover, immunohistochemical detection of TNF protein and microglial activation in tissue sections of transgenic but not of non-transgenic littermates indicates functional *in vivo* expression of the transgene. In addition, bioactive TNF could be detected on the cell membrane (Fig. 1, *D* and *E*) and in the supernatant of cultivated *NR2B/TNF* transgenic neurons (6×10^6 cells release 1 ng of soluble TNF in 24 h) but not in that of neurons from control littermates as determined by a standard bioassay (data not shown) (24, 25). Glutamate treatment had no effect on the

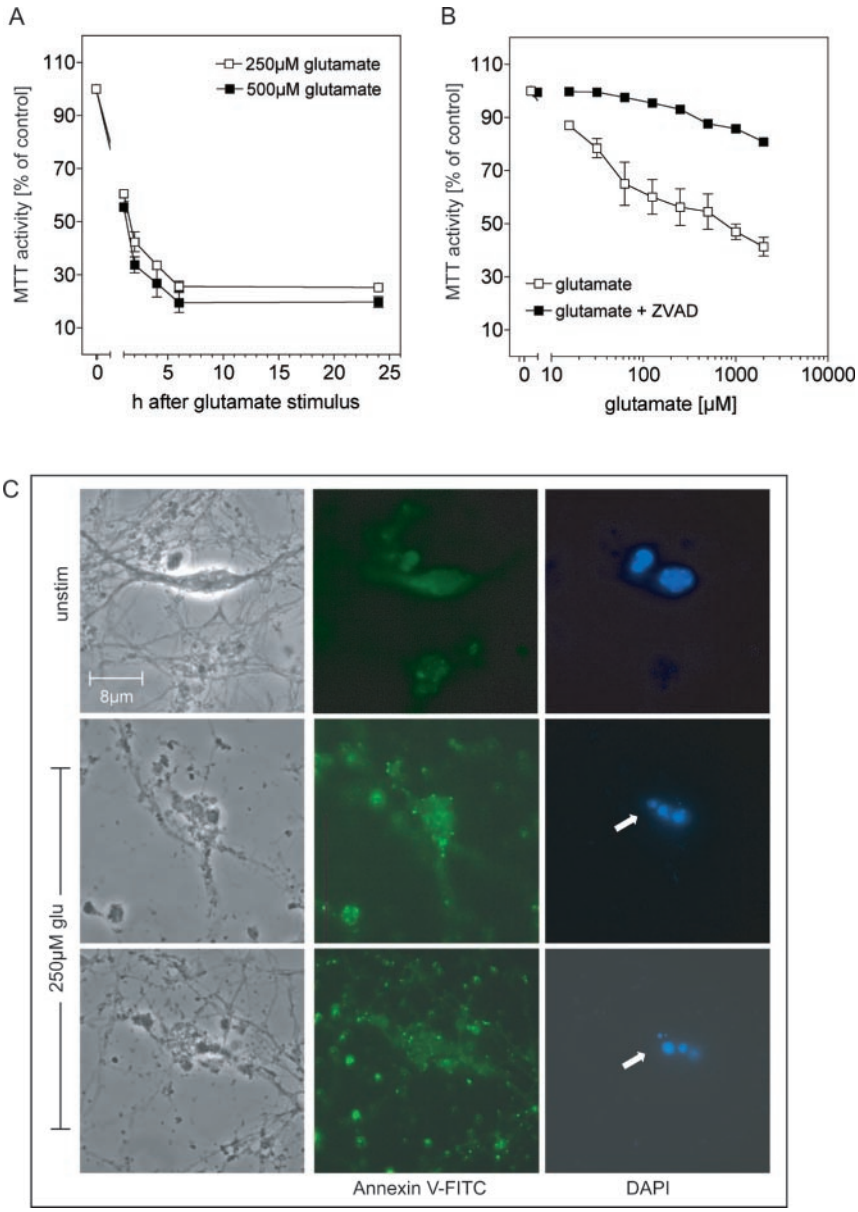

FIG. 2. **Glutamate-induced apoptosis in primary cortical wild type cultures.** *A*, 250 (□) and 500 (■) μM glutamate induces neuronal death in a time-dependent manner. Cell viability was assessed at the indicated times after a 1-h glutamate stimulus. *B*, dose-dependent neuronal cell death in the absence (□) and presence (■) of the caspase inhibitor Z-VAD (20 μM). Cell death in *A* and *B* was assessed by MTT assay ($n = 3$) as described under "Experimental Procedures." *C*, immunocytochemistry. DAPI (*blue fluorescence*) and annexin V (*green fluorescence*) staining of *in vitro* cultivated primary cortical neurons. *Upper panel* depicts unstimulated cells. *Lower panel* depicts neurons after treatment for 1 h with 250 μM glutamate. Glutamate-treated neurons exhibit membrane-localized punctated annexin V staining and nuclear fragmentation (*arrows*).

NR2B promoter activity as determined by semi-quantitative reverse transcription-PCR of *NR2B* mRNA levels in wild type neurons (data not shown).

TNF/TNFR2-mediated Signaling Protects Primary Cortical Neurons from Glutamate-induced Cell Death—Exposure of wild type murine primary cortical neurons to neurotoxic doses of glutamate lead to a time- and dose-dependent induction of cell death (Fig. 2, *A* and *B*), which was inhibited by the caspase inhibitor, Z-VAD-fmk (Fig. 2*B*). Furthermore, nuclear fragmentation, annexin V staining (Fig. 2*C*), and inhibition of neuronal cell death by MK801 (Fig. 3*A*), an inhibitor of the NMDA receptor, is indicative of NMDAR-mediated excitotoxic induction of apoptosis, which is in accordance with previous studies (26). Primary cortical neurons from *NR2B*/*TNF* transgenic animals, in contrast to wild type and TNFR1- ($TNFR1^{-/-}$) and TNFR2-deficient ($TNFR2^{-/-}$) cells, were found to be almost completely resistant to glutamate-triggered excitotoxicity (Fig. 3*A*). To mimic the conditions of continuous TNF expression of *TNF* transgenic animals, wild type and $TNFR^{-/-}$ neuronal cells were pretreated for 24 h with TNF. Under these conditions, resistance toward glutamate-induced apoptosis could be induced in wild type and, to an even greater extent, in $TNFR1^{-/-}$ neuronal cells (Fig. 3*B*). By contrast, $TNFR2^{-/-}$ neurons were not only more susceptible to glutamate-induced cell death but also died upon TNF exposure (Fig. 3*B*). TNF stimulation of neuronal cultures simultaneously or subsequent to glutamate addition was found to be non-protective (data not shown). These data indicate that the resistance of primary cortical neurons toward an excitotoxic stimulus requires a preceding TNF signal via TNFR2. Studying ischemia reperfusion-induced damage of the retina of $TNFR1^{-/-}$ mice, we have recently shown that the locally applied PI3K inhibitor, LY294002, aggravated neuronal degeneration (10). Therefore, we have investigated the role of PI3K in the glutamate-induced *in vitro* excitotoxicity model. The glutamate resistance of *NR2B*/*TNF* transgenic cortical neurons (Fig. 3*C*) and TNF-pretreated neurons of wild type or $TNFR1^{-/-}$ mice (Fig. 3*B*) was completely abolished by LY294002. Of note, although we found that protection of wild type and $TNFR1^{-/-}$ neurons required *in vitro* preincubation with TNF, the addition of the PI3K inhibitor as little as 30 min before the excitotoxic stimulus fully prevented the TNF-dependent survival of neurons (Fig. 3*B*). This finding indicates that in order to accumulate a protective TNF response, the TNFR2-mediated activation of a

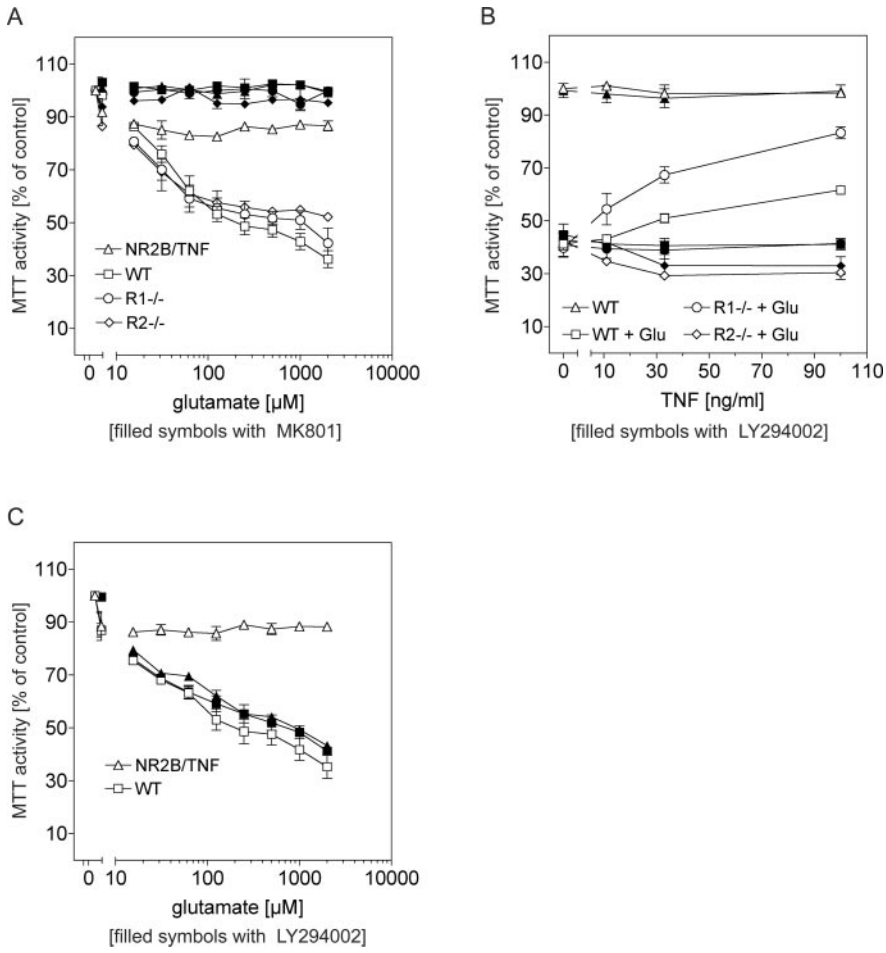

FIG. 3. **TNF mediates anti-apoptotic signals via TNFR2.** *A*, differences in sensitivity for glutamate-induced excitotoxicity of primary cortical neurons from different mouse lines in the absence (*open symbols*) or presence (*filled symbols*) of the NMDAR blocker MK801 (□ and ■, wild type (*WT*); △ and ▲, *NR2B/TNF*; ○ and ●, $TNFR1^{-/-}$ (*R1−/−*); ◇ and ◆, $TNFR2^{-/-}$ (*R2−/−*)). *B*, TNF dose-dependent protection of primary neuronal cultures after exposure to excitotoxic concentrations of glutamate (1 h, 250 μM) in the absence (*open symbols*) or presence (*filled symbols*) of the LY294002 (□ and ■, WT; ○ and ●, R1−/−; ◇ and ◆, R2−/−; △ and ▲, WT without glutamate to detect possible toxicity of the inhibitor (performed for all of the cell lines shown as an example for wild type cells)). *C*, the PI3K inhibitor, LY249002, can revert neuroprotection in *NR2B/TNF* transgenic neurons (△ and ▲, *NR2B/TNF*; □ and ■, WT; *open symbols*, without inhibitor; *filled symbols*, with LY249002). Cell death in *A*, *B*, and *C* was assessed by MTT assay ($n = 3$) as described under "Experimental Procedures."

PI3K pathway has to occur within a narrow time span before the excitotoxic insult.

Further evidence for distinct proapoptotic and anti-apoptotic pathways emanating from TNFR1 and TNFR2, respectively, was obtained by the use of TNFR subtype-specific agonistic antibodies (27), allowing selective stimulation of either receptor in wild type neurons. TNFR specificity of the antibodies was verified by analyzing reactivity on $TNFR1^{-/-}$ and $TNFR2^{-/-}$ neurons (data not shown). The TNFR1-specific agonist alone elicited neuronal cell death in wild type neurons in a dose-dependent manner and did not protect from glutamate-induced apoptosis (Fig. 4*A*). Conversely, selective stimulation of TNFR2 had no cytotoxic effect but instead led to an inhibition of neurotransmitter-triggered excitotoxicity (Fig. 4*B*). Again, the blocking of PI3K by LY294002 reverted the TNFR2-mediated protection (Fig. 4*B*), whereas the TNFR1-mediated cell death was not affected by the PI3K inhibitor (Fig. 4*A*). The critical involvement of PI3K signaling was confirmed by usage of wortmannin after selective TNFR2 stimulation (Fig. 4*C*) or TNF preincubation (data not shown).

NMDAR Signaling Enhances TNFR2-induced PKB/Akt Activation—PKB/Akt is considered to be a prominent and ubiquitous downstream target protein of PI3K (28). Because of the TNF- and PI3K-dependent protection of cortical neurons from glutamate-induced apoptosis, we investigated PKB/Akt activity during excitotoxic stimulation of untreated and TNF-pretreated wild type, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice as well as that of *NR2B/TNF* transgenic mice. Western blot analyses were performed with protein lysates from cortical neurons treated with TNF and/or glutamate in the presence or absence of a specific NMDAR blocker (MK801) or a selective PI3K inhibitor (LY294002). Intense and persistent PKB/Akt phosphorylation was detected in both $TNFR1^{-/-}$ and wild type neurons after TNF and glutamate co-stimulation (Fig. 5*A*, *lanes 4*, *8*, and *12*). This was partially inhibited by LY294002 in wild type cells and completely abolished in $TNFR1^{-/-}$ cells (Fig. 5*A*, *lanes 6*, *10*, and *14*). In $TNFR2^{-/-}$ neurons, only a weak transient PKB/Akt activation was detected upon glutamate and TNF treatment (Fig. 5*A*, *lanes 4*, *8*, and *12*). These data show that the TNF-induced persistent PKB/Akt phosphorylation is TNFR2-mediated and PI3K-dependent. Moreover, we reveal to date an unrecognized strong enhancement of this signal pathway by the glutamate-activated NMDAR.

In contrast to wild type, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ neurons, substantial levels of phospho-PKB/Akt were detected in *NR2B/TNF* transgenic neurons without *in vitro* TNF treatment (Fig. 5*B*, *lane 1*), which is in accordance with endogenous TNF expression in these neurons. As expected from the above experiments, PKB/Akt phosphorylation was PI3K-dependent at all of the time points analyzed (Fig. 5*B*, *lanes 4*, *7*, *10*, and *12*).

NMDAR Signaling Increases TNFR2-induced NF-κB Activation—NF-κB activation in primary cortical neurons was determined by immunofluorescence microscopy of nuclear translocated p65 (Fig. 6*A*) as well as by the EMSA (Fig. 6, *E* and *F*). The lone TNF treatment of $TNFR1^{-/-}$ or $TNFR2^{-/-}$ neurons revealed characteristic differences in NF-κB activation (Fig. 6*B*). Nuclear NF-κB translocation after stimulation of TNFR1 reached a maximum between 20 and 40 min (Fig. 6*B*, *white bars*), whereas TNFR2-mediated NF-κB translocation increased gradually, peaked after 4 h of stimulation, and was still discernable after 24 h (Fig. 6*B*, *black bars*). Wild type neurons, which express both TNFRs, exhibited intermediate translocation kinetics (Fig. 6*B*, *gray bars*). Upon glutamate stimulation

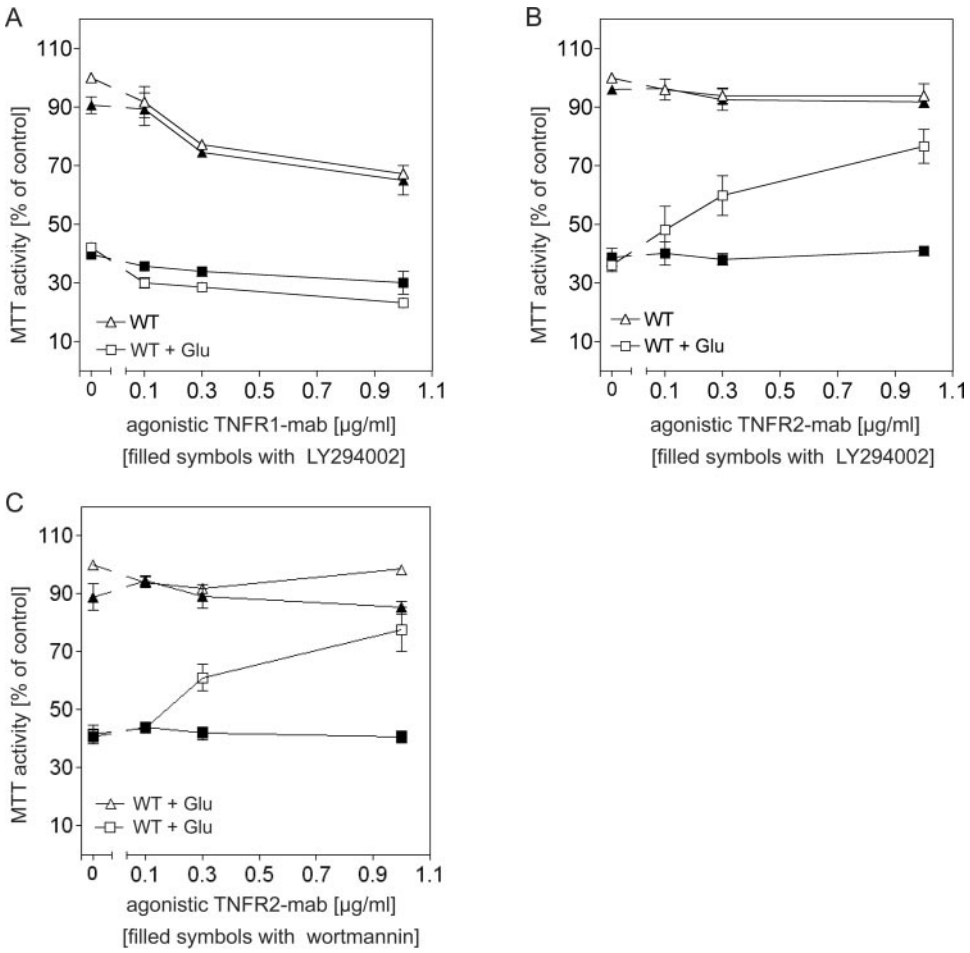

FIG. 4. **Agonistic TNFR antibodies mimic TNF preincubation in primary cortical wild type neurons.** *A*, dose-dependent toxicity of primary neuronal cultures by agonistic TNFR1-monoclonal antibody after exposure to excitotoxic concentrations of glutamate (1 h, 250 μM) in the absence (*open symbols*) or presence (*filled symbols*) of LY294002 (□ and ■, wild type (*WT*); △ and ▲, WT without glutamate to detect possible toxicity of the inhibitor). *B* and *C*, dose-dependent neuroprotection of primary neuronal cultures by agonistic TNFR2-monoclonal antibody after exposure to excitotoxic concentrations of glutamate (1 h, 250 μM) in the absence (*open symbols*) or presence (*filled symbols*) of LY294002 (*B*) or wortmannin (*C*) (□ and ■, WT; △ and ▲, WT without glutamate to detect possible toxicity of the inhibitor). Cell death in *A*, *B*, and *C* was assessed by MTT assay ($n = 3$) as described under "Experimental Procedures."

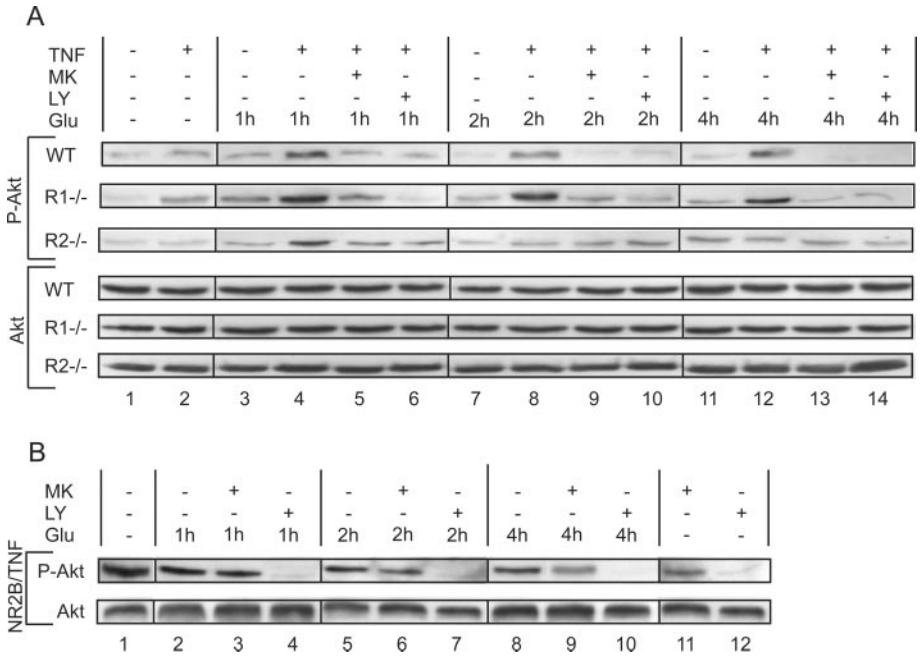

FIG. 5. **Western blot analysis of Akt in protein lysates from primary cortical cultures of different mouse lines treated with glutamate and inhibitors of NMDAR and/or PI3K.** *A*, detection of phosphorylated PKB/Akt (serine 473) and total PKB/Akt protein (in order to control equal protein loading) in wild type (*WT*), *TNFR1*$^{-/-}$ (*R1−/−*), and *TNFR2*$^{-/-}$ (*R2−/−*) neurons after TNF, MK801, or LY294002 preincubation and glutamate stimulation as indicated *B*. detection of PKB/Akt and its phosphorylated residue (serine 473) in *NR2B/TNF* transgenic neurons after MK801 or LY294002 preincubation and glutamate stimulation as indicated.

of TNF-pretreated (24 h) cultures, a strong enhancement of the residual low level NF-κB activity could be identified by nuclear translocation (Fig. 6*C*) and EMSA (Fig. 6*E*) in wild type and in *TNFR1*$^{-/-}$ neurons, whereas *TNFR2*$^{-/-}$ neurons showed no enhancement of NF-κB activation by glutamate (Fig. 6, *C*, *white bars*, and *E*, *lanes 2*, *4*, and *8*). Of note, upon glutamate treatment alone, no NF-κB activation was discerned (Fig. 6, *C* and *E*, *lanes 3*, *7*, and *11*). In TNF-pretreated wild type neurons, weak NF-κB activity (Fig. 6, *B*, *gray bars*, and *E*, *lane 2*) strongly increased after glutamate stimulation and persisted at elevated levels for at least 4 h (Fig. 6, *C*, *gray bars*, and *E*, *lanes 4*, *8*, and *12*). In these cells, the enhancement was strongly reduced by MK801 and LY294002 when assessed during the first 2 h (Fig. 6, *C*, *gray bars*, and *E*, *lanes 5*, *6*, *9*, and *10*) and NF-κB activity was no longer discernable by EMSA in LY294002-treated cultures at 4 h after glutamate stimulation (Fig. 6*E*, *lane 14*). These results indicate an important contribution of a TNFR2-dependent pathway for NF-κB activation in wild type neurons, in particular at later time points of glutamate exposure. *TNFR1*$^{-/-}$ neurons had comparable activation kinetics to wild type neurons with one remarkable difference. At every time point measured, NF-κB activation was completely inhibited by LY294002 (Fig. 6, *C*, *black bars*, and *E*, *lanes 6*, *10*, and *14*), clearly suggesting that, in cortical neuronal cells, TNFR2-mediated NF-κB activation is PI3K-dependent. Moreover, the virtually complete blocking of the enhancement of the TNF-induced NF-κB response by MK801 discloses the essential role of NMDAR co-activation in wild type and *TNFR1*$^{-/-}$ neurons (Fig. 6*E*, *lanes 5*, *9*, and *13*). By contrast, PI3K dependence of neuronal TNFR1-mediated NF-κB activation was apparently less stringent. For example, although the NF-κB response of TNF-pretreated *TNFR2*$^{-/-}$ neurons was partially LY 294002-sensitive 1 h post-glutamate treatment, it remained completely unaffected by LY294002 at 2 h (Fig. 6, *C* and *E*, *lanes 4 versus 6* and *8 versus 10*). Of greater relevance, EMSA confirmed that TNFR1-mediated NF-κB activation does not persist and lacks enhancement through NMDAR activation. Similar to PKB/Akt activity, *NR2B*/*TNF* transgenic neurons displayed basal NF-κB activity because of the constitutive expression of TNF (Fig. 6, *D* and *F*, *lane 1*). Additional glutamate stimulation enhanced nuclear NF-κB translocation (Fig. 6*D*) and its activation (Fig. 6*F*, *lanes 2*, *5*, and *8*), which could be partially reduced by MK801 (Fig. 6, *D* and *F*, *lanes 3*, *6*, and *9*) and fully abrogated by LY294002 2 and 4 h after glutamate stimulation (Fig. 6, *D* and *F*, *lanes 7* and *10*). Further, incubation of *NR2B*/*TNF* transgenic neurons with LY294002 inhibited endogenous NF-κB activation as well (Fig. 6*F*, *lane 12*), indicating that chronic TNF expression in these cells apparently imposes a bias on TNF signaling toward TNFR2 pathways. Supershift analysis using antibodies against p65, p50, and cRel identified p50 and p65 as the predominant NF-κB subunits (Fig. 6*G*) in all of the mouse strains tested. Although the latter experiment is in accordance with the view that both TNFR1 and TNFR2 use the canonical NF-κB pathway (9), the differential kinetics of activation and sensitivity toward PI3K inhibitors in primary cortical neurons indicate distinct upstream signal pathway usage of the two TNFRs. TNFR1-mediated induction of NF-κB is transient, whereas TNFR2 can induce a more lasting NF-κB activation. The latter is strictly PI3K-dependent and enhanced by co-stimulation of the ionotropic NMDA receptor. In contrast, the TNFR1-mediated NF-κB activation is less dependent on PI3K and not enhanced by NMDA receptor activation.

TNFR2-dependent Induction of IκB Degradation Is Sensitive to PI3K Inhibition—To assess the molecular level of PI3K/Akt-mediated NF-κB activation, IκBα phosphorylation and subsequent degradation were analyzed in *NR2B*/*TNF* transgenic or TNF-pretreated wild type, *TNFR1*$^{-/-}$, and *TNFR2*$^{-/-}$ neurons. In *TNFR1*$^{-/-}$ cells, the phosphorylation and depletion of IκB occurred earlier and was stronger as compared with wild type cells (Fig. 7*A*, *lanes 3–7*). LY294002 sensitivity of IκB phosphorylation and degradation was more pronounced in *TNFR1*$^{-/-}$ neurons as compared with wild type neurons (Fig. 7*A*, *lanes 10–14*). Consistent with the shorter NF-κB activation kinetics in *TNFR2*$^{-/-}$ neurons, only transient IκB phosphorylation and degradation were observed in these cells (Fig. 7*A*, *lanes 3–7*) that were largely LY204002-insensitive (Fig. 7*A*, *lanes 10–14*). *NR2B*/*TNF* transgenic neurons exhibited continuous (Fig. 7*B*, *lanes 2–6*) LY294002-sensitive (Fig. 7*B*, *lanes 8–12*) IκB phosphorylation and depletion due to the continuous expression of TNF. This finding is consistent with the basal activation of NF-κB observed in these cells (Fig. 6, *C* and *E*). LY294002 treatment significantly raised IκB levels and decreased the proportion of phosphorylated IκB in NR2B/TNF transgenic neurons (Fig. 7*B*, *lane 7*), indicating permanent PI3K-dependent signaling through TNFR2. The transient LY249002-insensitive reduction in IκB levels observed at around 60 min (Fig. 7*B*, *lane 11*) probably reflects concomitant TNFR1 activity and/or other TNFR2-independent signals. To verify specific proteasomal degradation, IκB levels were assessed in the presence of the proteasomal inhibitor, MG132, which prevented the stimulus-dependent reduction in IκB protein levels in all of the neuronal cultures (Fig. 7, *C* and *D*).

NF-κB Activation Is a Central Mediator of Neuroprotection—To scrutinize the protective role of NF-κB from the excitotoxic insult of cortical neurons, we blocked NF-κB by the use of three different inhibitors known to interfere with NF-κB activation at distinct levels, *i.e.* the assembly of the IKK complex via geldanamycin, the blocking of IκBα phosphorylation with BAY 11-7082, and the interference with proteasomal degradation of phosphorylated I-κB via MG-132 (29–32). Although MG-132, geldanamycin, and BAY 11-7082 treatment themselves showed a slight toxic effect in the *in vitro* neuronal culture system, all of these inhibitors completely blocked TNF-induced protection in *TNFR1*$^{-/-}$ and wild type cortical neurons (Fig. 8, *A–C*). *TNFR2*$^{-/-}$ neurons served as a negative control and revealed no influence of the applied inhibitors on the excitotoxic sensitivity of these cells (Fig. 8, *A–C*). Likewise, the resistance of *NR2B*/*TNF*-neuronal cultures toward glutamate-induced cell death could be abrogated by pharmacological intervention with NF-κB activation (Fig. 8*D*). These data provide strong evidence that NF-κB activation and thus transcription of NF-κB-dependent anti-apoptotic genes play a major role in TNF-mediated protection of neurons from excitotoxic insults.

DISCUSSION

We have recently shown the critical involvement of TNF and its receptors in retinal ischemia in which *TNFR1*-deficient animals were protected from ischemic lesions, whereas *TNFR2*-deficient animals developed severe pathology and enhanced neuronal loss compared with wild type animals. These data suggested a potential antagonistic function of TNFRs with respect to neuronal survival upon exogenous stress signals and/or tissue damage (10). Here we show that the same principle applies to cortical neurons and provide evidence for the underlying mechanism of the protective signal pathway using an *in vitro* model of glutamate-induced cell death of primary cortical neurons. Together, our previous *in vivo* studies (10) and the mechanistic *in vitro* studies presented here provide compelling evidence for a TNF-mediated anti-apoptotic pathway via TNFR2. First, a transgenic mouse line (*NR2B*/*TNF*) expressing TNF under the control of the murine NMDAR sub-

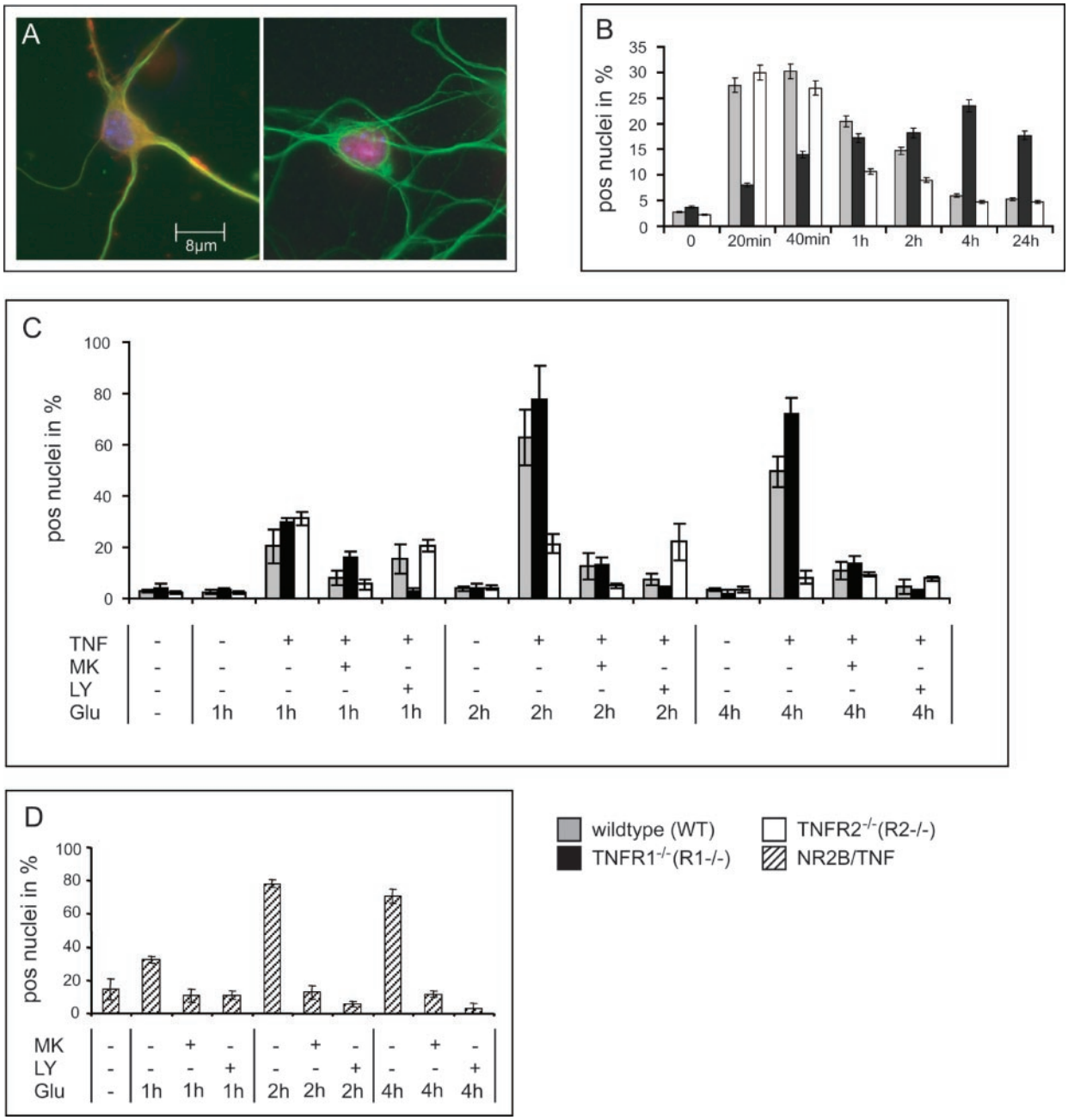

FIG. 6. **Nuclear p65 translocation in primary cortical neurons.** *A*, immunocytochemistry detection of p65 (*red fluorescence*, Alexa 546) and βIII-tubulin (*green fluorescence*, Alexa 488) in primary cortical wild type (*WT*) neurons. *Left picture* shows a TNF-pretreated neuron before stimulation with glutamate, and the *right picture* shows a TNF-pretreated neuron after stimulation with glutamate. *B*, kinetics of TNF-induced nuclear translocation of p65 (*dark gray bars*, wild type; *black bars*, *TNFR1*$^{-/-}$; *white bars*, *TNFR2*$^{-/-}$). *C*, kinetics of p65 translocation after TNF (24 h), MK801 (30 min), or LY294002 (30 min) preincubation and glutamate stimulation as indicated (*dark gray bars*, wild type; *black bars*, *TNFR1*$^{-/-}$; *white bars*, *TNFR2*$^{-/-}$). *D*, kinetics of p65 translocation in *NR2B/TNF* transgenic neurons after MK801 (30 min) or LY294002 (30 min) preincubation and glutamate stimulation as indicated. Nuclear translocation in *B*, *C*, and *D* was quantified by counting within a total of 400 cells/experiment the percentage of neuronal cells with NF-κB nuclear staining (as shown in *A* after treatment with TNF and glutamate). All of the bars depict the mean ± S.D. of three independent experiments, and the distribution of the data sets was analyzed by one-way ANOVA. NF-κB activation in primary cortical neurons. *E* and *F*, EMSAs for NF-κB activity in primary cortical cultures of different mouse lines. *E*, nuclear extracts of WT, *TNFR1*$^{-/-}$, and *TNFR2*$^{-/-}$ neurons after TNF, MK801, or LY294002 preincubation and glutamate stimulation as indicated were incubated with a ^{32}P-labeled NF-κB consensus oligonucleotide and analyzed as described under "Experimental Procedures." *F*, nuclear extracts from *NR2B/TNF* transgenic neurons after MK801 or LY294002 preincubation and glutamate stimulation as indicated were incubated with a ^{32}P-labeled NF-κB consensus oligonucleotide and analyzed as described under "Experimental Procedures." *G*, supershift for NF-κB. Nuclear extracts of WT, R1−/−, R2−/−, and *NR2B/TNF* transgenic neurons were preincubated with p65, p50, or cRel antibodies (1 μg/ml) and analyzed as described under "Experimental Procedures."

unit *NR2B* promoter exerts, *in vivo*, locally restricted modest TNF activity neither causing brain inflammation nor any apparent pathological signs or neurological deficits.[2] Cortical neurons isolated from these *NR2B/TNF* mice were significantly protected from glutamate-induced apoptosis. Second, we have used the TNF pretreatment of wild type and TNFR-deficient primary cortical neurons to mimic the phenotype observed with neurons from TNF transgenic mice and demonstrate that TNF-treated neurons from wild type and

[2] L. Marchetti, M. Klein, K. Schlett, K. Pfizenmaier, and U. L. M. Eisel, unpublished data.

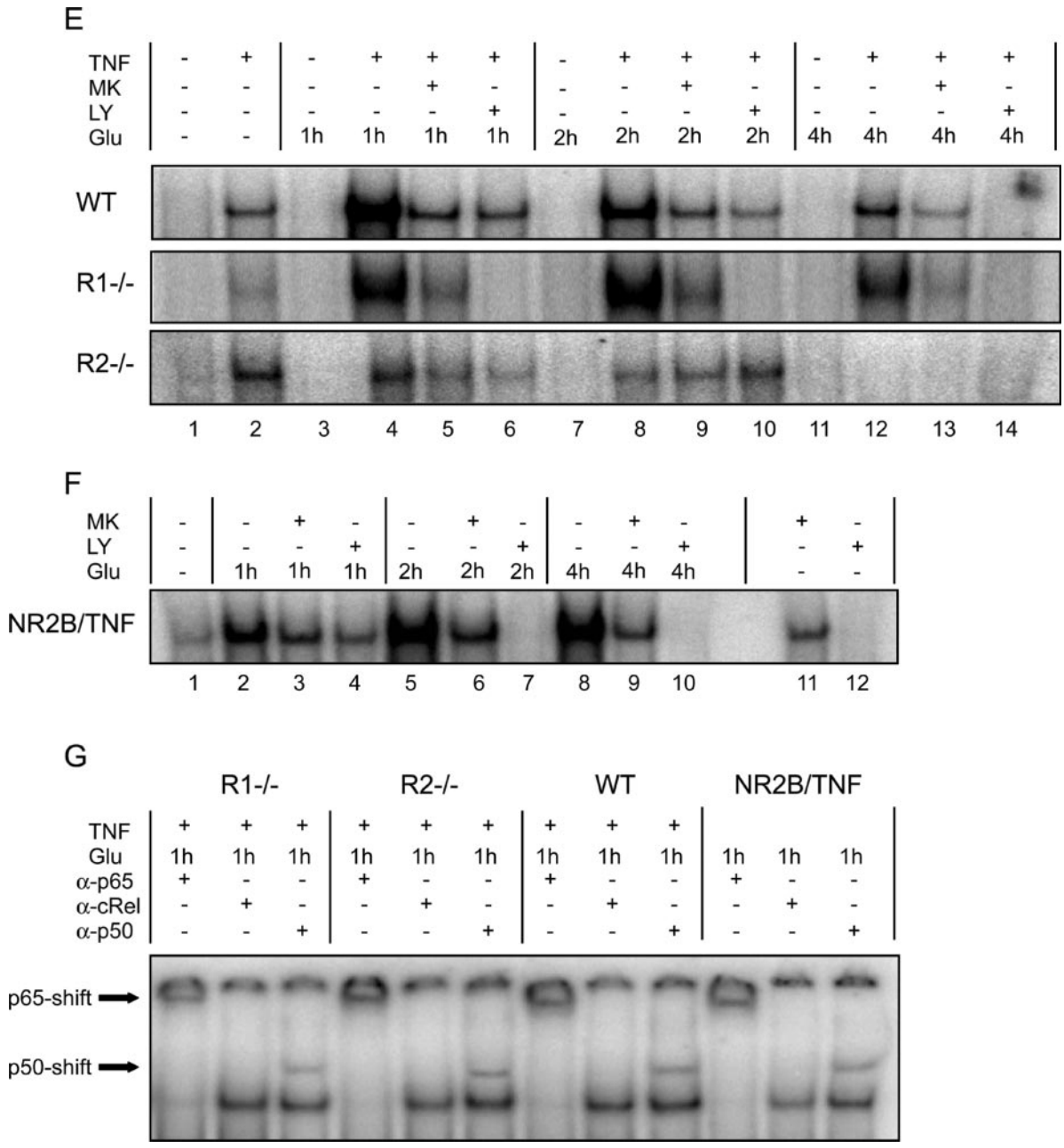

FIG. 6—*continued*

TNFR1$^{-/-}$ mice were significantly protected, whereas primary neurons from *TNFR2*$^{-/-}$ mice were susceptible to cell death. Third, we show by kinetic analyses of NF-κB activation and by pharmacological inhibition of signal pathway components in the TNFR knock-out mouse strains that neuroprotection is mediated via a TNFR2-PI3K-Akt-NF-κB pathway in which the duration of NF-κB activation is the critical determinant in mounting resistance toward excitotoxic insults.

The high concentration of soluble recombinant murine TNF required to achieve significant protection *in vitro* is already indicative of an involvement and critical role of TNFR2, which has at 37 °C a lower affinity for soluble TNF and a reduced signal competence as compared with TNFR1, thus necessitating higher TNF concentrations for signaling (33). Moreover, the primary neurons derived from the TNF transgenic mouse line that are protected from excitotoxicity express TNF not only in its processed form but also at the neuronal cell membrane, a prerequisite for efficient TNFR2 activation. Both an autotropic and a juxtatropic signaling of membrane-expressed TNF are conceivable. As cortical neurons co-express both TNFRs, we conclude that, under constitutive membrane TNF expression in the transgenic neurons, the neuroprotective TNFR2 pathway is dominant over TNFR1 signals. Direct support for differential roles of the two TNFRs in signaling neuronal survival comes from the use of TNFR-specific agonistic antibodies where TNFR2- but not TNFR1-specific agonists promoted protection from excitotoxicity. A differential role of TNFR1 and TNFR2 has also been reported for hippocampal neurons responding to TNF alone with TNFR1 inducing both NF-κB activation and cell death, whereas TNFR2 was found to stimulate p38 mitogen-activated protein kinase (11). However, this study neither addressed the functional significance of TNFR2-mediated p38 activation nor resolved the underlying mechanism of apoptosis dominance in these cells in the presence of an apparently strong NF-κB activation (11), which is considered to play an important role in neuroprotection (34). As an example, the

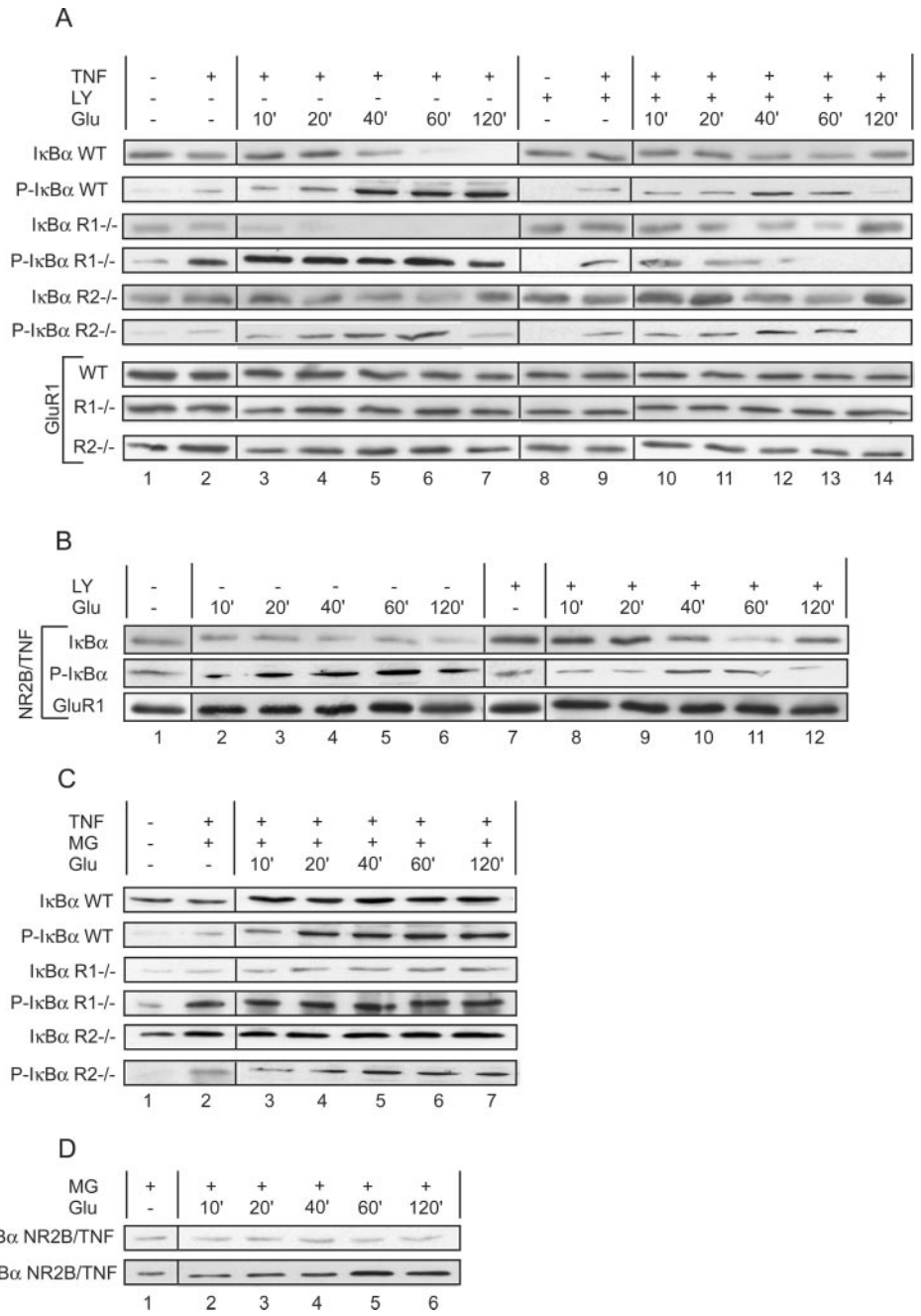

FIG. 7. **Western blot analyses of Iκb in lysates from primary cortical cultures of different mouse lines treated with glutamate and inhibitors of PI3K.** *A*, detection of Iκb degradation and phosphorylation in primary cortical wild type (*WT*), *TNFR1*$^{-/-}$ (*R1−/−*), and *TNFR2*$^{-/-}$ (*R2−/−*) neurons after TNF or LY294002 preincubation and glutamate stimulation as indicated. *B*, detection of Iκb degradation and phosphorylation in *NR2B/TNF* transgenic neurons after LY294002 preincubation and glutamate stimulation as indicated. Immunoblotting of the glutamate receptor 1 protein (*GluR1*) was performed in order to control equal protein loading for all of the cell lines, because GluR1 levels were constant during stimulation. *C*, verification of Iκb phosphorylation in the presence of the proteasomal inhibitor MG-132 in primary cortical WT, *TNFR1*$^{-/-}$, *TNFR2*$^{-/-}$, and neurons after TNF preincubation and glutamate stimulation as indicated. *D*, verification of Iκb phosphorylation in the presence of the proteasomal inhibitor, MG-132, in primary cortical NR2B/TNF transgenic neurons after glutamate stimulation as indicated. Equal protein loading for *C* and *D* was assessed by GluR1 immunoblotting (data not shown).

cellular inhibitors of apoptosis proteins, well known NF-κB-target gene(s) up-regulated by TNF, have been recently identified as facilitators in down-regulating apoptosis sensitivity within neurons (35, 36). The prominent role of NF-κB in protection from neurodegeneration has recently received further support from a transgenic model characterized by forebrain-specific, inducible, and complete ablation of NF-κB activity by I-κB superrepressor (37). Hippocampal slice cultures from these NF-κB-deficient animals presented with strongly increased lesions upon excitotoxic stress.

In accordance with the important role of NF-κB in neuroprotection, here we show that upon *in vitro* glutamate exposure of TNF-treated cortical neurons, a PI3K-dependent PKB/Akt phosphorylation was ensued by NF-κB activation. The kinetics of both PKB/Akt and NF-κB activation support a TNFR2-initiated signal pathway to NF-κB in a PI3K-dependent manner and the involvement of PKB/Akt. A potential role of other kinases located downstream of PI3K that could contribute to NF-κB activation in response to TNF, *e.g.* PKCζ, cannot be formally ruled out. However, *in vivo*, PKCζ is apparently a

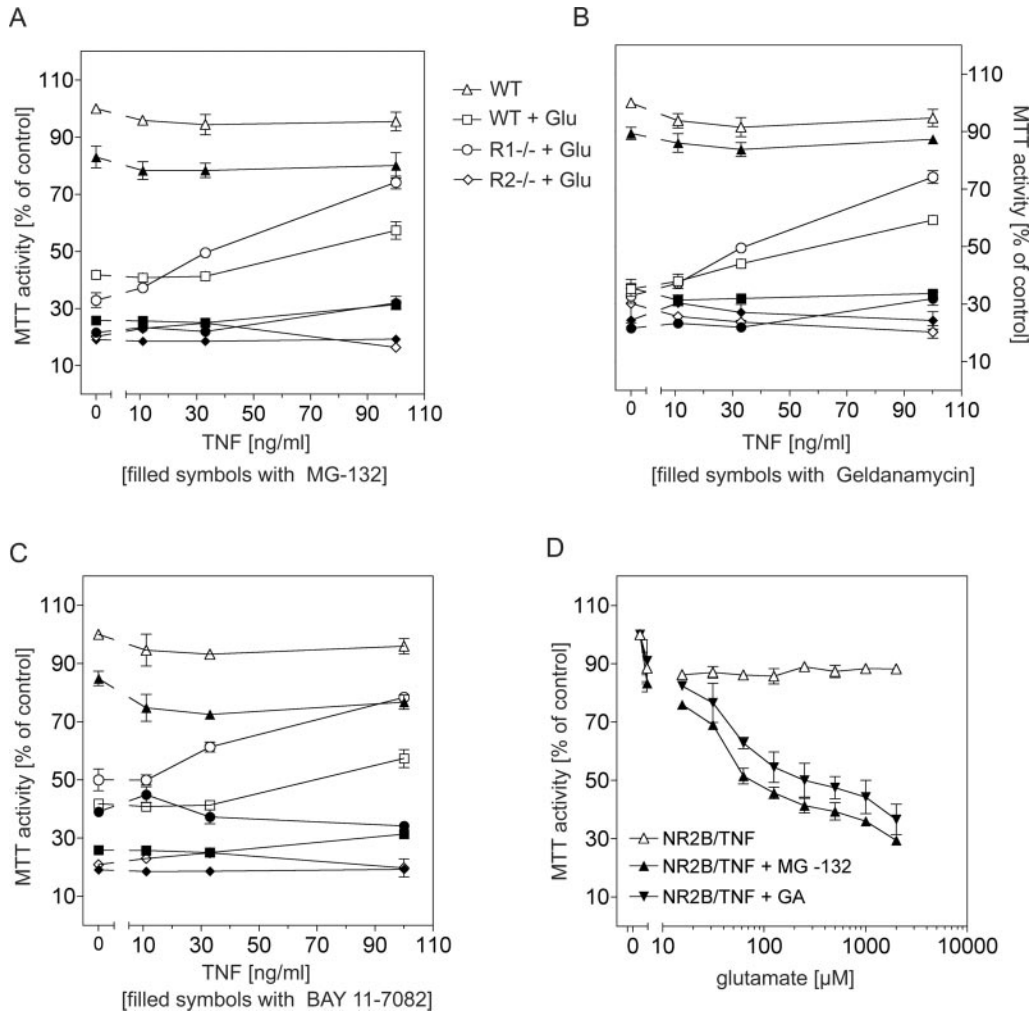

FIG. 8. **NF-κB activation is important for glutamate resistance.** *A*, TNF-mediated neuroprotection in the absence (*open symbols*) or presence of the proteasome inhibitor, MG-132 (*filled symbols*). □ and ■, wild type (*WT*); ○ and ●, *TNFR1*$^{-/-}$ (*R1−/−*); ◇ and ◆, *TNFR2*$^{-/-}$ (*R2−/−*); △ and ▲, WT without glutamate to detect possible toxicity of the inhibitor (performed for all of the cell lines shown as an example for wild type cells). *B*, TNF-induced resistance against glutamate induced cell death in the absence (*open symbols*) or presence of the Hsp90 inhibitor, geldanamycin (*filled symbols*). □ and ■, WT; ○ and ●, R1−/−; ◇ and ◆, R2−/−; △ and ▲, WT without glutamate to detect possible toxicity of the inhibitor (performed for all of the cell lines shown as an example for wild type cells). *C*, TNF-mediated neuroprotection in the absence (*open symbols*) or presence of the IκB phosphorylation inhibitor, BAY 11-7082 (*filled symbols*). □ and ■, WT; ○ and ●, R1−/−; ◇ and ◆, R2−/−; △ and ▲, WT without glutamate to detect possible toxicity of the inhibitor (performed for all of the cell lines shown as an example for wild type cells). *D*, glutamate resistance of NR2B/TNF transgenic neurons can be reverted by both MG132 and geldanamycin. △, *NR2B/TNF*; ▲, *NR2B/TNF* plus MG-132; ▼, *NR2B/TNF* plus geldanamycin. Cell death in *A*, *B*, *C*, and *D* was assessed by MTT assay ($n = 3$) as described under "Experimental Procedures."

tissue-selective NF-κB activator with its dominant function (with respect to NF-κB activation) in lung tissues (39). Moreover, the main action of PKCζ in the context of TNF-mediated NF-κB activation has now been recognized to be at the level of RelA transactivation (38). In contrast, our data indicate that PI3K operates at a level upstream of IκB degradation, *i.e.* probable at the level of IKK activation or IκB itself as shown in other cell systems (39–43). Examples with established cell lines show that PKB/Akt may serve as an IKK kinase in response to TNF (42). More recent data indicate that IKKα is the prime target of PKB/Akt phosphorylation in the heterotrimeric IKK complex, resulting in IKKβ activation and subsequent IkBα phosphorylation (47). Our data with primary neurons reveal PKB/Akt activation in a TNFR2-specific manner. Strict PI3K dependence of TNFR2-mediated IκB phosphorylation and activation of p50/p65 as well as neuroprotection is in full agreement with a cell context-specific function of PKB/Akt in the canonical NF-κB pathway.

Of note, NF-κB activation via TNFR1-induced pathways did not protect cortical neurons from glutamate-induced apoptosis, which is in accordance with data from hippocampal neurons responding to TNF only (11). Interestingly, our data reveal that, in primary neurons, TNFR1- and TNFR2-induced pathways differ with respect to the kinetics and duration of NF-κB activation with the TNFR1 inducing a rapid but transient NF-κB response and TNFR2 generating a more persistent response, still discernible after several hours when stimulated with TNF alone. As an implication, the duration of NF-κB activation is critical to achieve significant tissue protection. The usage of different upstream activators of NF-κB by TNFR1 and TNFR2 is a probable explanation for the observed differential kinetics and duration of the NF-κB response. Indeed, it becomes increasingly apparent that the cell-specific quantitative composition with signal proteins contributing to a complex and multi-regulated pathway critically determines response phenotypes. For NF-κB activation, for example, a multitude of combinatorial possibilities exist, constituting canonical and non-canonical pathways, and comprise of several potential upstream IKK-activating kinases and several IκB isoforms with differential sensitivity to degradation and feedback regulation

(45–48). The individual composition of a cell with the relative amounts of each of these signaling molecules may not only influence the potential linkage to receptor proximal signals but also the kinetics and duration of NF-κB activation. Our data provide a clear example that differences in the initial signal input (TNFR1 *versus* TNFR2 pathway), although activating the same intracellular key molecule (NF-κB), are translated into a completely different signal output (cell death *versus* survival) by virtue of a differential duration of the active state of this critical molecule.

Blocking the NMDA receptor with MK801 lead to a reduction of PKB/Akt and NF-κB activation, revealing an as of yet unknown cross-talk among a cytokine receptor, TNFR2, and the ionotropic NMDA receptor. It can be speculated that this positive cooperation with respect to PKB/Akt and NF-κB activation may serve as a regulative feedback to limit cytotoxic signals emanating from overactivated NMDARs. In favor of this reasoning, only persistent NF-κB activation is associated with protection from cell death. Our data show that this requires a cooperative action of TNFR2 with the ionotropic NMDA receptor, which meet above or at the level of PI3K-PKB/Akt. The molecular mechanisms of the NMDAR-TNFR2 cooperation are not yet resolved. It is apparent from sequential stimulation studies (data not shown) that the signals emanating from TNFR2 have to precede the excitotoxic insult. At first glance, this would be in accordance with strictly TNF-mediated acquisition of an apoptosis-resistant state of the cell via an NF-κB-dependent transcriptional response without a contribution of NMDAR signals. However, pharmacological inhibition of the PI3K pathway just before glutamate stimulation not only blocked the TNFR2-dependent and NMDAR-enhanced stimulation of PKB/Akt and NF-κB but also fully abrogated protection from apoptosis. This finding suggests that the acute signal cross-talk between the two receptor systems is essential for establishing a protective response. We propose that sustained PKB/Akt activity ensures prolonged NF-κB activity and counterbalances the negative feedback of NF-κB-dependent IκB expression typical for TNFR1-mediated signals causing transient NF-κB activity (9). Indeed, neurons protected from excitotoxicity showed consistently reduced/lower IkB levels as compared with sensitive ones. In support of our reasoning, a recent study has linked calcium-induced pathways via PI3K and Akt toward NF-κB activation in neuronal cells (51).

A critical function of other PI3K-PKB/Akt pathways in neuronal survival has been proposed, and several downstream targets aside from NF-κB, in particular Bad, potentially interfere with apoptotic signals and could be involved in neuroprotection (49). For example, calcineurin-induced Bad dephosphorylation seems to play an important role in calcium-induced apoptosis within hippocampal neurons, an effect feasible in glutamate-treated cortical neurons as well (50). However, a dominant role for other PKB/Akt substrates, including Bad, in TNF-induced protection of cortical neurons from excitotoxicity seems unlikely because selective inhibitors of NF-κB activation operating downstream of PKB/Akt, such as a specific inhibitor of IκBα phosphorylation (31, 32) or inhibition of proteasomal IκB degradation (30), prevented protection from glutamate-induced cell death.

TNF is up-regulated in a number of neurodegenerative disorders including Alzheimer's disease, stroke, and multiple sclerosis. However, its function remained to be defined because both neurotoxic and neuroprotective effects during disease pathogenesis have been described previously (50–55). The *TNF* transgenic mouse line described here clearly shows that restricted neuronal TNF expression is *a priori* not harmful. To the contrary, the response phenotype of primary neurons isolated from these mice suggests that TNF expression conditions the neurons toward resistance to excitotoxic insults. Support for the *in vivo* relevance of a TNFR2-mediated neuroprotective pathway was previously obtained by us in a model of retinal ischemia (10). We now have preliminary evidence in a model of cerebral ischemia that *NR2B/TNF* transgenic mice display reduced lesions as compared with wild type animals.[3]

In conclusion, here our data present that TNF mediates neuroprotection via TNFR2 and that NMDAR co-signaling provides new mechanistic insights and sheds new light into the role of TNF in the brain. We propose that TNF functions as an important regulatory cytokine in the central nervous system with differential signaling through the two distinct TNFRs determining its contribution to degenerative and regenerative processes. Thus, depending on the type and state of disease, environmental/external factors, cellular composition of the affected tissue, and intracellular availability of TNFR signaling components, TNF may function to aggravate or ameliorate disease. A complete understanding of the differential signals that are initiated by TNFR1 and TNFR2 in neurons and of their cross-talk with other pathways that are important for neuronal viability is essential in order to derive therapeutically effective strategies. From our study, we conclude that selective triggering of TNFR2 could be a novel therapeutic strategy in neurodegenerative diseases because it actively induces an anti-apoptotic state in neurons by cooperative action with neurotransmitter receptor signals. This mechanism elucidated here can now be exploited for therapeutic gain in combination with or independent of drugs interfering with apoptotic signal pathways.

Acknowledgments—We thank Horst Bluethmann for kindly providing the *TNFR1* and *TNFR2* knock-out mice. We gratefully acknowledge Hycult Biotechnology, for generous supply with murine TNFR1- and TNFR2-specific antibodies. We thank Bernd Kirchherr for excellent technical assistance, Jan Bauer and Hans Lassmann for help with immunohistochemistry, and Lesley Probert for critically reading the paper.

REFERENCES

1. Fleischmann, R., Iqbal, I., Nandeshwar, P., and Quiceno, A. (2002) *Expert Opin. Drug Saf.* **25,** 173–197
2. Mueller, C. (2002) *Immunology* **105,** 1–8
3. Lou, J., Lucas, R., and Grau, G. E. (2001) *Clin. Microbiol. Rev.* **14,** 810–820
4. Tyor, W. R., Wesselingh, S. L., Griffin, J. W., McArthur, J. C., and Griffin, D. E. (1995) *J. Acquired Immune Defic. Syndr. Hum. Retrovirol.* **9,** 379–388
5. Perry, R. T., Collins, J. S., Wiener, H., Acton, R., and Go, R. C. (2001) *Neurobiol. Aging* **22,** 873–883
6. Probert, L., and Akassoglou, K. (2001) *Glia.* **36,** 212–219
7. Clark, W. M., and Lutsep, H. L. (2001) *Expert Opin. Biol. Ther.* **1,** 227–237
8. Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995) *Cell* **83,** 793–802
9. Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003) *Cell Death Differ.* **10,** 45–65
10. Fontaine, V., Mohand-Said, S., Hanoteau, N., Fuchs, C., Pfizenmaier, K., and Eisel, U. (2002) *J. Neurosci.* **22,** 1–7
11. Yang, L., Lindholm, K., Konishi, Y., Li, R., and Shen, Y. (2002) *J. Neurosci.* **22,** 3025–3032
12. Shen, Y., Li, R., and Shiosaki, K. (1997) *J. Biol. Chem.* **272,** 3550–3553
13. Eisel, U., Reynolds, K., Riddick, M., Zimmer, A., Niemann, H., and Zimmer, A. (1993) *EMBO J.* **12,** 3365–3372
14. Keffer, J., Probert, L., Cazlaris, H., Georgopoulos, S., Kaslaris, E., Kioussis, D., and Kollias, G. (1991) *EMBO J.* **10,** 4025–4031
15. Klein, M., Pieri, I., Uhlmann, F., Pfizenmaier, K., and Eisel, U. (1998) *Gene (Amst.)* **208,** 259–269
16. Mosmann, T. (1983) *J. Immunol. Methods* **65,** 55–63
17. Probert, L., Akassoglou, K., Pasparakis, M., Kontogeorgos, G., and Kollias, G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 11294–11298
18. Akassouglou, K., Probert, L., Kontogeorgos, G., and Kollias, G. (1997) *J. Immunol.* **158,** 438–445
19. Akassoglou, K., Bauer, J., Kassiotis, G., Pasparakis, M., Lassmann, H., Kollias, G., and Probert, L. (1998) *Am. J. Pathol.* **153,** 801–813
20. Kassiotis, G., Bauer, J., Akassoglou, K., Lassmann, H., Kollias, G., and Probert, L. (1999) *J. Immunol.* **29,** 912–917
21. Watanabe, M., Inoue, Y., Sakimura, K., and Mishina, M. (1993) *J. Comp. Neurol.* **338,** 377–390

[3] U. L. M. Eisel and H. Petit, unpublished data.

22. Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B., and Seeburg, P. H. (1994) *Neuron* **12,** 529–540
23. Sasner, M., and Buonanno, A. (1996) *J. Biol. Chem.* **271,** 21316–21322
24. Eskandari, M. K., Nguyen, D. T., Kunkel, S. L., and Remick, D. G. (1990) *Immunol. Investig.* **19,** 69–79
25. Green, S., Dobrjansky, A., and Chiasson, M. A. (1982) *J. Natl. Cancer Inst.* **68,** 997–1003
26. Lee, J. M., Zipfel, G. J., and Choi, D. W. (1999) *Nature* **399,** Suppl. 6738, A7–A14
27. Tacchini-Cottier, F., Vesin, C., Redard, M., Buurman, W., and Piguet, P. F. (1998) *J. Immunol.* **160,** 6182–6186
28. Cantley, L. C. (2002) *Science* **296,** 1655–1657
29. Lewis, J., Devin, A., Miller, A., Lin, Y., Rodriguez, Y., Neckers, L., and Liu, Z. G. (2000) *J. Biol. Chem.* **275,** 10519–10526
30. Hellerbrand, C., Jobin, C., Iimuro, Y., Licato, L., Sartor, R. B., and Brenner, D. A. (1998) *Hepatology* **27,** 1285–1295
31. Pierce, J. W., Schoenleber, R., Jesmok, G., Best, J., Moore, S. A., Collins, T., and Gerritsen, M. E. (1997) *J. Biol. Chem.* **272,** 21096–21103
32. Petegnief, V., Saura, J., de Gregorio-Rocasolano, N., and Paul, S. M. (2001) *Neuroscience* **104,** 223–234
33. Grell, M., Wajant, H., Zimmermann, G., and Scheurich, P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 570–575
34. Yabe, T., Wilson, D., and Schwartz, J. P. (2001) *J. Biol. Chem.* **276,** 43313–43319
35. Fotin-Mleczek, M., Henkler, F., Samel, D., Reichwein, M., Hausser, A., Parmryd, I., Scheurich, P., Schmid, J. A., and Wajant, H. (2002) *J. Cell Sci.* **115,** 2757–2770
36. Wiese, S., Digby, M. R., Gunnersen, J. M., Gotz, R., Pei, G., Holtmann, B., Lowenthal, J., and Sendtner, M. (1999) *Nat. Neurosci.* **2,** 978–983
37. Fridmacher, V., Kaltschmidt, B., Gaudeau, B., Ndiaye, D., Rossi, F. M., Pfeiffer, J., Kaltschmidt, C., Israel, A., and Memet, S. (2003) *J. Neurosci.* **23,** 9403–9408
38. Duran, A., Diaz-Meco, M. T., and Moscat, J. (2003) *EMBO J.* **22,** 3910–3918
39. Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J. F., Camacho, F., Diaz-Meco, M. T., Rennert, P. D., and Moscat, J. (2001) *Mol. Cell* **8,** 771–780
40. Sandra, F., Matsuki, N., Takeuchi, H., Ikebe, T., Kanematsu, T., Ohishi, M., and Hirata, M. (2002) *Cell Signal.* **14,** 771–778
41. Madrid, L. V., Mayo, M. W., Reuther, J. Y., and Baldwin, A. S., Jr. (2001) *J. Biol. Chem.* **276,** 18934–18940
42. Romashkova, J. A., and Makarov, S. S. (1999) *Nature* **401,** 86–90
43. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) *Nature* **401,** 82–85
44. Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995) *Cell* **24,** 573–582
45. Hoffmann, A., Levchenko, A., Scott, M. L., and Baltimore, D. (2002) *Science* **298,** 1241–1245
46. Schmidt, C., Peng, B., Li, Z., Sclabas, G. M., Fujioka, S., Niu, J., Schmidt-Supprian, M., Evans, D. B., Abbruzzese, J. L., and Chiao, P. J. (2003) *Mol Cell.* **12,** 1287–1300
47. Gustin, J. A., Ozes, O. N., Akca, H., Pincheira, R., Mayo, L. D., Li, Q., Guzman, J. R., Korgaonkar, C. K., and Donner, D. B. (2004) *J. Biol. Chem.* **279,** 1615–1620
48. Gosh, S., and Karin, M. (2002) *Cell* **109,** S81–96
49. Brunet, A., Datta, S. R., and Greenberg, M. E. (2001) *Curr. Opin. Neurobiol.* **11,** 297–305
50. Wang, J., Asensio, V. C., and Campbell, I. L. (2002) *Curr. Top. Microbiol. Immunol.* **265,** 23–48
51. Lilienbaum, A., and Israel, A. (2003) *Mol. Cell. Biol.* **23,** 2680–2698
52. Shohami, E., Ginis, I., and Hallenbeck, J. M. (1999) *Cytokine Growth Factor Rev.* **1,** 119–130
53. Eisel, U. L. M. (2002) *Curr. Top. Microbiol. Immunol.* **265,** 49–62
54. Kim, G. M., Xu, J., Xu, J., Song, S. K., Yan, P., Ku, G., Xu, X. M., and Hsu, C. Y. (2001) *J. Neurosci.* **21,** 6617–6625
55. Kassiotis, G., and Kollias, G. (2001) *J. Exp. Med.* **193,** 427–434